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Abstract: This work reports the isolation, purification, and anti-inflammatory evaluation of compounds from the plant species *Ageratina pichinchesis*. Using open-column chromatography, 11 known compounds were purified, whose chemical structures were elucidated by nuclear magnetic resonance techniques (1D and 2D). All compounds were evaluated in the in vitro model of RAW 264.7 mouse macrophage cells measuring nitric oxide inhibition as a determination of the anti-inflammatory effect. Outstandingly, the compound betuletol 3-O-β-glucoside (**11**) exhibited inhibition of nitric oxide with an IC_{50} of 75.08 ± 3.07% at 75 μM, additionally, it inhibited the secretion of interleukin 6 (IL-6) and activation of nuclear factor (NF-kβ). These results show that compound 11 could be considered as a potential anti-inflammatory agent in suppressing the expression of NF-kβ target genes such as the proinflammatory pathway and inducible nitric oxide synthase (iNOS). Consequently, the medicinal use attributed in traditional medicine to the *A. pichinchensis* species is due to the diversity of compounds that the plant biosynthesizes, demonstrating that its effect relates to anti-inflammatory effects associated with compound **11**.

Keywords: betuletol 3-*O*-β-glucoside; *A. pichinchensis*; anti-inflammatory activity

1. Introduction

The plant species *A. pichinchensis* (Kunth) R.M. King & Ho. Steal. (Asteraceae), is native to Mexico and grows in different states [1–4]. Particularly, in the state of Morelos it is popularly known, as "axihuitl" which is mainly used for treating stomach pain, and respiratory, gastrointestinal, and skin infections [5–7].

Scientific studies carried out in different biological evaluation models reveal that the aerial parts of *A. pichinchensis* exhibit activity against onychomycosis, tinea pedis, gastroprotective and healing effects, and even inhibit the in vitro proliferation of keratinocytes [8–13]. The main compounds associated with this type of biological effect are chromenes, furans, terpenes, essential oils, and glycosylated flavonoids [10–15]. However, the anti-inflammatory effect of the plant species has not been reported.

Inflammation is a response of the immune system whose stimulation can be caused by infections or stress and is associated with multiple diseases and triggers numerous biochemical, immunological, and cellular reactions [16–19]. During the inflammatory process, mediating substances are released

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in the nervous system that generate pain, among which are interleukins that induce genes that encode enzymes and other chemical species that contribute to the inflammatory response [20–23].

In particular, the enzyme nitric oxide synthase (iNOS) catalyzed the formation of the free radical nitric oxide (NO) in a gaseous state that stimulates vasodilation and cannot be stored [24–26]. It should be noted that the participation of NO plays a defensive role, acting as an immunoregulatory and antimicrobial agent due to its ability to generate DNA damage, inhibit energy metabolism enzymes, oxide proteins, and peroxidize membrane lipids of pathogenic agents, however, in high concentrations of NO exert the same effects on the cells of inflamed organs, exacerbating the damage and consequently the inflammatory process [27–30].

The iNOS enzyme can be elicited in different cell types, such as macrophages, hepatocytes, neutrophils, etc. [31–33], which generates a large amount of NO that can be toxic, which is why NO functions as a pro-inflammatory molecule [34–36]. In a chronic phase of inflammation, the inflammatory signal can be magnified by the production of molecules such as nuclear transcription factor kappa β (NF-kβ), TNF-α, and interferon-gamma (IFN-γ) which promote transcription of iNOS, generating large amounts of NO, greater vasodilation, edema, and plasma exudation that can even damage the DNA double helix [37–40]. High production of NO can be associated with the development of diseases such as Alzheimer's, cardiovascular, rheumatoid arthritis, pulmonary fibrosis, diabetes, and cancer, among others [41–46]. This is why the iNOS enzyme is essential in the inflammatory process and allows us to understand the mechanism of action of active ingredients from medicinal plants [47–49].

Particularly, this study reports the anti-inflammatory activity of chemical compounds isolated from aerial parts (leaves and flowers) of *A. pichinchensis* on the inhibition of NO in RAW 264.7 mouse macrophage cells induced with lipopolysaccharides (LPS).

2. Materials and Methods

Compounds **1**-**11** were characterized using spectroscopic techniques and mass spectrometry; for compounds **1**, **3**, **4**, **6**, **7,** and **8**, a Varian Unity Inova 200 MHz equipment was used; Compounds **2**, **5**, **9,** and **10** were analyzed on a Varian Mercury Plus 400 MHz—ID3 spectrometer, for these compounds $CDCl_3$ was used, compound **11** was dissolved in DMSO-d_6, the spectra of ^{1}H, ^{13}C, DEPT, COSY, HSQC, and HMBC were obtained on a Bruker AVANCE III HD 500 MHz Spectrometer (Billerica, MA, USA). FABMS spectra were obtained using a JEOL-AX 505HA mass spectrometer. Optical rotation was measured in $CHCl_3$ on a Perkin Elmer 241 digital polarimeter at 25 °C. Melting points were determined on a Prendo apparatus [50–52]. Compounds 1-11 were purified using column chromatography (CC), silica gel 60 (70-230 and 230-400, mesh) as stationary phase, and thin layer chromatography (silica gel 60 F_{254}, Merck) to monitor the separation of the compounds, which were visualized using a solution of Ce $(SO_4)_2$ $(NH_4)_2SO_4$ $.2H_2O$.

2.1. Plant material

Complete plant *A. pichinchensis* was collected by M.B. Mariana Sánchez Ramos in the town of San Juan Tlacotenco, municipality of Tepoztlán, Morelos, Mexico, in April 2018 (19°00′43.88′′ N, 99.05′38.66′′ W). The plant was prepared (pressed) and taken to the HUMO Herbarium of the Autonomous University of the State of Morelos (UAEM). The Biol. Gabriel Flores Franco identified this species, registered with voucher number 33913 [53].

2.2. Extraction and isolation

Leaves were selected from the collected plants and dried at room temperature. This material (1.085 kg) was successfully extracted with AcOEt three times (each time with 4 L of the solvent).

The AcOEt extracts obtained were concentrated to dryness by distillation under reduced pressure using a rotary evaporator, obtaining 20.3 g of residue. Fractionation of the EtOAc extract by open CC (silica gel, 70–230 mesh; 10 cm i.d. × 60 cm) was performed with a gradient system of *n*-hexane-EtOAc 80:20 to 0:100, collecting 35 fractions of 300 mL each.

Based on TLC analysis, these fractions were grouped according to their chemical profile into four groups; AP-1A (fractions 1–5, *n*-hexane: EtOAc 80:20, 4.12 g), AP-1B (fractions 6–19, *n*-hexane: EtOAc 60:40 and 40:60, 5.23 g), AP-1C (fractions 20–32, *n*-hexane: EtOAc 20:80, 5.94 g) and AP-1D (fractions 33–35, EtOAc 100 %, 3.52 g). AP-1A contains aliphatic esters, fatty acids, and *O*-methylenecalinol (1, 48 mg) as the main product, as well as AP-1D of sugars. The groups AP-1B and AP-1C were subjected to column chromatography using silica gel (70–230 mesh). The AP-1B fraction (5.23 g) was adsorbed on 4.9 g of silica gel and placed in a glass column (70 cm high and 3.5 cm in diameter) packed with 157 g of silica gel. Elution was carried out using a gradient system, *n*-hexane:Ethyl acetate (100:00→50:50) and 164 fractions were obtained in 50 mL. These were concentrated and monitored by TLC, grouped in 6 groups of fractions. The AP-1B-1 group (fractions 1–39, 0.306 g, *n*-hexane: ethyl acetate 95:05 to 85:15) was purified and identified as *O*-methylencecalinol (**1**, 32 mg) as the main product; from AP-1B-2 (fractions 40–69, 3.32 g, *n*-hexane:ethyl acetate 80:20 to 70:30) they were identified 7-hydroxiencecalin (**3**, 7 mg) and 8-hydroxiencalin (**4**, 6 mg); the compounds encecalin (**2**, 16 mg), 3,5-diprenyl-4-hydroxyacetophenone (**5**, 32 mg) and (+)-β-eudesmol (**6**, 8 mg) were isolated from the AP-1B-3 group (fractions 70–99, 0.391 g, *n*-hexane: ethyl acetate 65:35 to 55:45); The AP-1B-4 group (fractions 100–129, 0.821 g, *n*-hexane: ethyl acetate 50:50 to 40:60) led to the identification of the compounds (+)-β-eudesmol (**6**, 7 mg), dehydrospeletone (**8**, 12 mg) and speletone (**7**, 14 mg); through successive chromatography of the AP-1B-5 group (fractions 130–149, 0.427 g, *n*-hexane: ethyl acetate 35:75 to 25:75) were isolated encecalinol (**9**, 28 mg) and 5-acetyl-3β-angeloxy-2β-(1-hydroxyisopropyl)-2,3-dihydrobenzofuran (**10**, 36 mg).

O-methylencecalinol (**1**)

Colorless oil, ^{1}H-NMR (200 MHz, $CDCl_3$), δ_H : 6.95 (1H, s, H-5), 6.32 (1H, s, H-8), 6.26 (1H, d, *J* = 9.6 Hz, H-4), 5.43 (1H, d, *J* = 10 Hz, H-3), 4.61 (1H, q, *J*= 12.8, 6.8 Hz, H-11), 3.75 (3H, s, -OMe), 3.21 (3H, , OMe), 1.40 (3H, s, CH_3-13), 1.39 (3H, s, CH_3-14), 1.34 (3H, d, *J*=6.8 Hz, CH_3-12). ^{13}C-NMR (50 MHz, $CDCl_3$). δ_C : 157.70 (C-7), 153.25 (C-10), 127.76 (C-3), 124.06 (C-5), 122.30 (C-4), 120.02 (C-6), 114.19 (C-9), 99.43 (C-8), 76.55 (C-2), 72.97 (C-11), 56.59 (-OMe-C11), 56.03 (OMe-C-7), 28.32 (C-13), 28.26 (C-14), 22.65 (C-12), these data match those in the literature [12,54]. Spectra of ^{1}H and ^{13}C-NMR are in Figures S1 and S2.

Encecalin (**2**)

Yellow oil; ^{1}H-NMR (400 MHz, $CDCl_3$), δ_H : 7.23 (1H, s, H-5), 6.27 (1H, s, H-8), 6.12 (1H, d, *J* = 9.8 Hz, H-4), 5.44 (1H, d, *J* = 9.7 Hz, H-3), 3.55 (3H, s, -OMe), 2.10 (3H, s, CH_3-12), 1.20 (6H, s, CH_3-13 and CH_3-14). ^{13}C-NMR (100 MHz, $CDCl_3$). δ_C : 197.68 (C-11), 161.37 (C-7), 158.28 (C-10), 128.94 (C-5), 128.36 (C-3), 121.36 (C-4), 120.42 (C-6), 114.22 (C-9), 99.76 (C-8), 77.21 (C-2), 55.72 (MeO-), 32.26 (C-12), 26.68 (C-13), 28.32 (C-14), these data match those in the literature [55,56]. Spectra of ^{1}H and ^{13}C-NMR are in Figures S3 and S4.

Euparoriochromene (**3**)

Yellow needles; mp: 78–80 ◦C; ^{1}H-NMR (200 MHz, $CDCl_3$), δ_H : 7.21 (1H, s, H-5), 6.24 (1H, s, H-8), 6.21 (1H, d, *J* = 10 Hz, H-4), 5.41 (1H, d, *J* = 10 Hz, H-3), 2.49 (3H, s, CH_3-12), 1.39 (6H, s, CH_3-13 and CH_3-14). ^{13}C-NMR (50 MHz, $CDCl_3$). δ_C : 198.13 (C-11), 162.18 (C-7), 159.18 (C-10), 128.68 (C-5), 128.53 (C-3), 126.14 (C-6), 122.08 (C-4), 114.38 (C-9), 104.26 (C-8), 77.43 (C-2), 32.12(C-12), 28.86 (C-13) and 28.78 (C-14), these data match those in the literature [57]. Spectra of ^{1}H and ^{13}C-NMR are in Figures S5 and 62.

6-acethyl-8-hydroxy-2,2-dimethylchromene (**4**)

White powder; mp: 98 °C; ^{1}H-NMR (200 MHz, $CDCl_3$), δ_H : 7.62 (1H, d, *J*= 1.2 Hz, H-7), 7.43 (1H, d, *J*= 1.2 Hz, H-5), 6.34 (1H, d, *J* = 10.2 Hz, H-4), 5.71 (1H, d, *J* = 10 Hz, H-3), 2.56 (3H, s, CH_3-12) and 1.43 (6H, s, CH_3-13 and CH_3-14). ^{13}C-NMR (50 MHz, $CDCl_3$). δ_C : 202.98 (C-11), 165.23 (C-8), 160.68 (C-10), 128.61 (C-6), 128.32 (C-3), 125.34 (C-9), 122.24 (C-4), 118.38 (C-5), 114.31 (C-7), 78.26 (C-2), 28.58 (C-13 and C-14) and 26.38 (C-12), these data match those in the literature [58–60]. Spectra of ^{1}H and ^{13}C-NMR are in Figures S7 and S8.

3.5-diprenyl-4-hydroxyacetophenone (**5**)

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Crystalline solid; mp: 93-95 °C; ^{1}H-NMR (400 MHz, $CDCl_3$), δ_H : 7.44 (2H, s, H-2 y H-6), 5.98 (1H, s, OH), 5.31(2H, m, H-2′ y H-2′′), 3.37 (2H, d, J = 7.1 Hz, H-1′y H-1′′), 2.48 (3H, d, J = 19.8 Hz, CH_3-8), 1.74 (12H, d, J = 11.1 Hz, CH_3-4′, CH_3-4′′y CH_3-5′, CH_3-5′′). ^{13}C-NMR (100 MHz, $CDCl_3$), δ_C : 197.52 (C-7), 157.59 (C-4), 135.28 (C-1), 130.15 (C-31, C-3′′), 129.02 (C-2, C-6), 127.30 (C-3, C-5), 121.58 (C-2′, C-2′′), 29.82 (C-1′, C-1′′), 26.51 (C-8), 25.99 (C-5′′, C-4′) y 18.10 (C-4′′, C-5′), these data match those in the literature [55,61]. Spectra of ^{1}H and ^{13}C-NMR are in Figures S9 and S10.

β-eudesmol (**6**)

White amorphous solid; mp = 78-79 °C; ^{1}H-NMR (200 MHz, $CDCl_3$), δ_H : 4.70 (1H, d, J = 2 Hz, H-15b), 4.43 (1H, d, J = 1.6 Hz, H-15a), 2.38 (2H, m, Hs-3), 1.98 (1H, m, H-10), 1.25-1.62 (6H, m,CH_2-5, CH_2-7 and CH_2-8), 1.00 -1.36 (5H, m, CH_2-1, CH_2-2 and CH-6), 1.39 (3H, s, CH_3-11), 1.40 (3H, s, CH_3-12), 0.69 (3H, s, CH_3-14). ^{13}C-NMR (50 MHz, $CDCl_3$), δ_C : 152.41 (C-4), 105.14(C-15), 72.23 (C-11), 49.58 (C-10), 49.42 (C-6),42.12 (C-3), 41.86 (C-1), 41.12 (C-8), 36.89 (C-9), 26.76 (C-12 and C-13), 24.73 (C-5), 23.45 (C-2), 22.76 (C-7) and 16.24 (C-14), these data match those in the literature [55,62,63]. Spectra of ^{1}H and ^{13}C-NMR are in Figures S11 and S12.

Speletone (**7**)

Colorless oil; ^{1}H-NMR (200 MHz, $CDCl_3$), δ_H : 8.09 (1H, d, J = 2.4 Hz, H-2), 7.78 (1H, dd, J = 2.2, 7.8 Hz, H6), 6.84 (1H, d, J = 8.4 Hz, H-5), 3.60 (3H, s, OMe), 2.67 (2H, d, J = 7.8 Hz, H-10), 2.33 (3H, s, CH_3 -8), 221 (1H, m, H-11), 0.78 (6H, d, J = 6.8 Hz, CH_3 -12 and CH_3 -13), these data match those in the literature [55,64]. Spectra of ^{1}H -NMR is in Figure S13.

Dehydrospeletone (**8**)

Colorless oil; ^{1}H-NMR (200 MHz, $CDCl_3$), δ_H: 8.16 (1H, d, J = 2.2 Hz, H-2), 8.06 (1H, dd, J = 2.2, 7.8 Hz, H6), 6.98 (1H, d, J = 8.2 Hz, H-5), 6.56 (1H, q, H-10), 3.92 (3H, s, OMe), 2.55 (3H, s, CH_3 -8), 2.22 (3H, s,CH_3 -12) and 1.95 (3H, s, CH_3 -13), these data match those in the literature [64,65]. Spectra of ^{1}H-NMR is in Figure S14.

Encecalinol (**9**)

Yellow oil; $[a]_D^{25}$: -77° (c 0.92, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$), δ_H: 6.95 (s, H-5), 6.37 (s, H-8), 6.27 (d, J = 9.7 Hz, H-4), 5.47 (d, J = 9.8 Hz, H-3), 5.02 (q, J = 6.5 Hz, H-13), 3.81 (s, MeO-), 1.47 (d, J = 6.5 Hz, CH_3-12), 1.42 (6H, s, CH_3-13 and CH_3-14). ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C: 157.26 C-7), 153.18 (C-10), 127.64 (C-3), 125.76 (C-6),123.83 (C-5), 122.02 (C-4), 113.75 (C-9), 99.53 (C-8), 77.06 (C-2), 65.53 (C-11), 55.45 (MeO-7), 28.04 (C-13), 27.96 (C-14), 22.86 (CH_3-12), these data match those in the literature [55,56,66]. Spectra of ^{1}H and ^{13}C-NMR are in Figures S15 and S16.

5-acetyl-3β--angeloyloxy-2β-(1-hydroxyisopropyl)-2,3-dihydrobenzofurane (**10**)

Yellow oil; $[a]_D^{25}$= +47 (c= 0.8, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$), δ_H : 7.86 (d, J = 2.1 Hz, H-4), 7.82 (dd, J = 8.6, 2.2 Hz, H-6), 6.88 (d, J = 8.6 Hz, H-7), 6.24 (m, H-3′), 5.96 (d, J = 7.5 Hz, H-3), 3.88 (d, J = 7.5 Hz, H-2), 2.52 (s, CH_3-14), 2.04 (dq, J = 7.3, 1.5 Hz, CH_3-4′), 1.93 (p, J = 1.5 Hz, CH_3-5′), 1.51 (s, CH_3-11), 1.35 (s, CH3-12). ^{13}C-NMR (100 MHz, $CDCl_3$), δ_C : 196.49 (C-13), 169.43 (C-1′), 157.12 (C-9), 141.17 (C-3′), 132.48 (C-5), 130.42 (C-4), 129.76 (C-6), 126.81(C-2′), 119.53 (C-8), 117.49 (C-7), 79.60 (C-10), 74.03 (C-2), 71.73 (C-3), 26.29 (C-12), 25.88 (C-14), 20.58 (C-5′), 19.73 (C-13) and 16.12 (C-4′), these data match those in the literature [12,55]. Spectra of ^{1}H and ^{13}C-NMR are in Figures S17 and S18.

Selected flowers from the collected plants and were dried in the shade at room temperature. The plant material (256.7 g) was successively extracted with MeOH: H_2O (95:05 v/v) three times (each time with 4 L of the solvent). The hydroalcoholic extracts obtained were concentrated to dryness by distillation under reduced pressure using a rotary evaporator, obtaining 6.7 g of residue. Fractionation of the hydroalcoholic extract by open CC (silica gel, 70–230 mesh; 10 cm i.d. × 60 cm) was performed with a gradient system of *n*-hexane-CH_2Cl_2: MeOH 90:10:00 to 100 % MeOH. Fractions of 100 mL were obtained (53 fractions). Based on TLC analysis, these fractions were grouped according to their chemical profile into two main groups: AP-M-1A (1-32, 2.02 g) and AP-M-1B (33-53, 3.98 g). The groups were subjected to column chromatography using silica gel (70–230 mesh). The AP-M-1A fraction was adsorbed on 3 g of silica gel and placed in a glass column (80 cm high and 3.5 cm in diameter) packed with 70 g of silica gel; elution was carried out using a gradient system, *n*-hexane: CH_2Cl_2 (100:00→80:20), 37 fractions were obtained 100 mL. These were concentrated and monitored by TLC and grouped into three groups of fractions. The AP-M-1A-1 group (fractions 1–

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17, 0.87 g), AP-M-1A-2 (fractions 18-26, 0.606 g) and AP-M-1A-3 (fractions 27-32, 0.87 g). The three groups of fractions were subjected to successive purification processes, obtaining from the AP-M-1A-1 fraction as the main product *O*-methylencecalinol (**1**, 32.4 mg), from the AP-M-1A-2 group the encecalin (**2**, 24 mg) and 3, 5-diprenyl-4-hydroxyacetophenone (**5**, 16 mg) and the AP-M-1A-3 group speletone (**7**, 14 mg), dehydrospeletone (**8**, 11 mg) and encecalinol (**9**, 17 mg) were purified. Likewise, through successive chromatographers using a gradient system (CH_2Cl_2: MeOH 95:05 to 80:20), betuletol 3-*O*-β-glucoside (**11**, 64 mg) was purified.

Betuletol 3-O-β-glucoside (**11**)

^{1}H-NMR (500 MHz, DMSO-d6); yellow amorphous solid; mp: 152-154 °C; δ_H : 12.58 (1H, s, OH), 8.00 (2H, d, *J* = 8.9 Hz, H-2′ H-6′), 6.90 (1H, s, H-8), 6.82 (2H, d, *J* = 8.9 Hz, H-3′ H-5′), 5.25 (1H, d, *J* = 7.7 Hz, H-1′′), 3.85 (3H, s, OMe-H-4′), 3.70 (3H, s, OMe-H-6), 3.58-3.56 (1H, ddd, *J* = 9.7, 7.7, 4.7 Hz, H-4′′), 3.32-3.31 (1H, m, H-5′′), 3.37-3.36 (1H, m, H-3′′), 3.47-3.45 (1H, m, H-2′′), 3.33-3.29 (1H, m, H-6′′). ^{13}C-NMR (125 MHz, DMSO-d_6), δ_C : 177.86, (C-4), 160.13, (C-4′), 158.80 (C-7), 156.78, (C-2), 151.81 (C-5), 151.68 (C-9), 133.29 (C-3), 131.76 (C-6), 128.57 (C-2′, C-6′), 120.65 (C-1′), 115.56 (C-3′, C-5′), 105.36 (C-10), 101.99 (C-1′′), 91.82 (C-8), 73.95 (C-5′′), 73.54 (C-3′′), 74.13 (C-2′′), 70.59 (C-4′′), 60.63 (C-6′′), 60.64 (C-OMe-6), 56.97 (C-OMe-4′), these data match those in the literature [67,68]. Spectra of ^{1}H and ^{13}C, DEPT and HMBC NMR are in Figures S19 to S22. $C_{23}H_{25}O_{12}$ (MSFAB$^+$ m/z = 493) MS is in figure S23.

2.3. In vitro anti-inflammatory activities

For the biological assay, the following reagents were purchased: Murine macrophage cell line RAW 264.7 of ATCC were purchased (Georgetown, Washington, DC, USA), Fetal bovine serum (FBS), Advanced DMEM/F12 medium, GlutaMAX (Gibco, Waltham, MA USA), GlutaMAX), DMSO, indomethacin, etoposide, LPS (Escherichia coli serotype 055:B5), $NaNO_2$, H_3PO_4, ursolic acid (UA) and sulfanilamide (Sigma Aldrich, St. Louis, MO, USA), salt MTS (Promega Co), Kit IL-6 (Pharmingen, USA).and MycoZapMT Plus-CL antibiotic (Lonza).

2.3.1. Cell Viability Assay (RAW 264.7)

Murine macrophage cell line RAW 264.7 (ATCC) was grown in ADVANCED DMEM/F12 medium supplemented with 2 mM glutamine and 3.5% heat-inactivated FBS, without antibiotics. Cells were at 37 °C in a humidified incubator in an atmosphere of 5% CO2. RAW 264.7 cells were grown in 24-well plates at a density of approximately 1X10^5 cells per well. The compounds were dissolved in dimethyl sulfoxide (DMSO) and filtered through 0.45 μm cellulose membranes [53].

Cell viability was determined by MTS (Promega) assay. Cells were seeded in 96-well plates (2 x 10^5 cells/well) in 100 μL of medium and incubated for 24 h. The cells were treated with various concentrations (0–75 μM) of test compounds (**1-11**), vehicle (DMSO, 0.5%, v/v) or etoposide (positive control, 68 μM) and incubated for 20 h. After treatment, 20 μL of MTS was added to each well, and the cells were incubated for another 4 h. The optical density was measured at 490 nm on a microplate reader (Epoch microplate spectrophotometer, Bio-Tek) [69,70].

2.3.2. Stimulation of RAW 264.7 Cells with LPS

RAW 264.7 macrophages were cultured at 2 X 10^5 cells/mL density in 96-well plates for 24 h. The cells were then pre-treated with compounds (**1-5**, **7-11**) at noncytotoxic concentrations (0-75 μM), or vehicle (DMSO, 0.5%, v/v), or indomethacin (84 μM) for 2 h, and after two h, were incubated with LPS (10 μg/mL) for 20 h to stimulate NO production. Cell-free supernatants were collected and were kept at -20 °C until NO quantification. The suppressive effect of compounds on NO production was assessed using the Griess reagent [71–73].

2.3.3. Determination of NO concentration

Nitrite released in the culture medium was measured according to Griess reaction. Briefly, 50 μL of each cell culture supernatant was mixed with 100 μL of Griess reagent (50 μL of 1%

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sulfanilamide and 50 µL of 0.1% N-(1-naphtyl) ethylenediamine dihydrochloride in 2.5 % phosphoric acid), for 10 min at room temperature. The optical density at 540 nm (OD_{540}) was measured with a microplate reader and nitrite concentration in the samples was calculated by comparison with the OD_{540} of a standard curve of $NaNO_2$ prepared in fresh culture medium [45,74,75].

2.3.4. Analysis of Pro-Inflammatory Cytokine IL-6

The anti-inflammatory effect of compound 11 was analyzed in RAW 264.7 cells induced by LPS. Cells were seeded in 96-well plates at a density of 3 X 10^4 cells/well incubated for 24 h. The cells were expressed with 1 microgram/ml of LPS and compound 11 at concentrations 37.5, 75, and 150 microgram/mL for 24 h. IL-6 levels in the cell culture medium were measured using an Elisa kit following the manufacturer's instructions (Pharmingen, USA) [76,77].

2.4. Treatment of RAW-Blue Cells with LPS

RAW-Blue Cells (3 × 10^4 cells/well) were plated in 96-well plates in 0.1 mL of DMEM/F12 medium supplemented with 10% FBS, 1% MycoZap, and 200 µg/mL Zeocin, and incubated for 24 h at 37 °C with 5% CO_2. Macrophages were then incubated with the test compounds for 2 hours at the maximum noncytotoxic concentration that showed an inhibitory effect on NO production to subsequently be incubated with LPS at 10 µg/mL (for wells with compounds and 100% control). stimulus) as a pro-inflammatory stimulus and without LPS (negative control) at 37 °C for 20 h to stimulate NF-κβ activation. Finally, cell-free supernatants were collected and used fresh to determine NF-κβ activation.

The QUANTI-Blue assay allows the detection of NF-κβ/AP-1 in the RAW-Blue system. Alkaline phosphatase activity (AP) was used as an indicator of NF-κβ activation in the supernatants (culture medium) by using QUANTI-Blue (InvivoGen), according to the instructions provided by the manufacturer [78,79].

2.5. Statistical analysis

The results shown were obtained at least by three independent experiments and are presented as means ± SDs. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. All statistical analyses were performed using GraphPad Prism, version 6.0 software. p values < 0.05 were considered to indicate statistical significance [80].

3. Results and discussion

3.1. Chemical Composition

The ethyl acetate (leaves) and hydroalcoholic (flowers) extracts obtained from the species *A. pichinchensis* were subjected to successive purification processes using the column chromatography technique and normal phase silica gel, a gradient elution system. -hexane: ethyl acetate (100:00→50:50 v/v) and *n*-hexane:CH_2Cl_2:CH_3OH (90:10:00→00:00:100 v/v). After several successive purification processes, pure compounds were obtained and identified by analysis of their spectroscopic data and by comparison with literature data. From the ethyl acetate extract (leaves) were identified: *O*-methylencecalinol (**1**) [55], encecalin (**2**)[12,55], eupatoriochromene (**3**) [81], 6-acetyl-8-hydroxy-2,2-dimethylchromene (**4**) [82–84], 3, 5-diprenyl-4-hydroxyacetophenone (**5**) [53,55], (+)-β-eudesmol (**6**) [50,51,55], espeletone (**7**) [52,55], dehydrospeletone (**8**)[52,53], encecalinol (**9**) [54,55,86], 5-acetyl-3β-angeloyloxy-2β-(1-hydroxyisopropyl)-2,3-dihydrobenzofurane (**10**)[12] and of the hydroalcoholic extract (flowers), in addition to the compounds (**1**, **2**, **5** and **6**), betuletol 3-*O*-β-glucoside (**11**) was identified[55,69].

Compounds (**3**, **4**, **7,** and **11**) were isolated from this plant for the first time and investigated the abilities of compounds **1**-**11** (Figure 1) to inhibit NO production.

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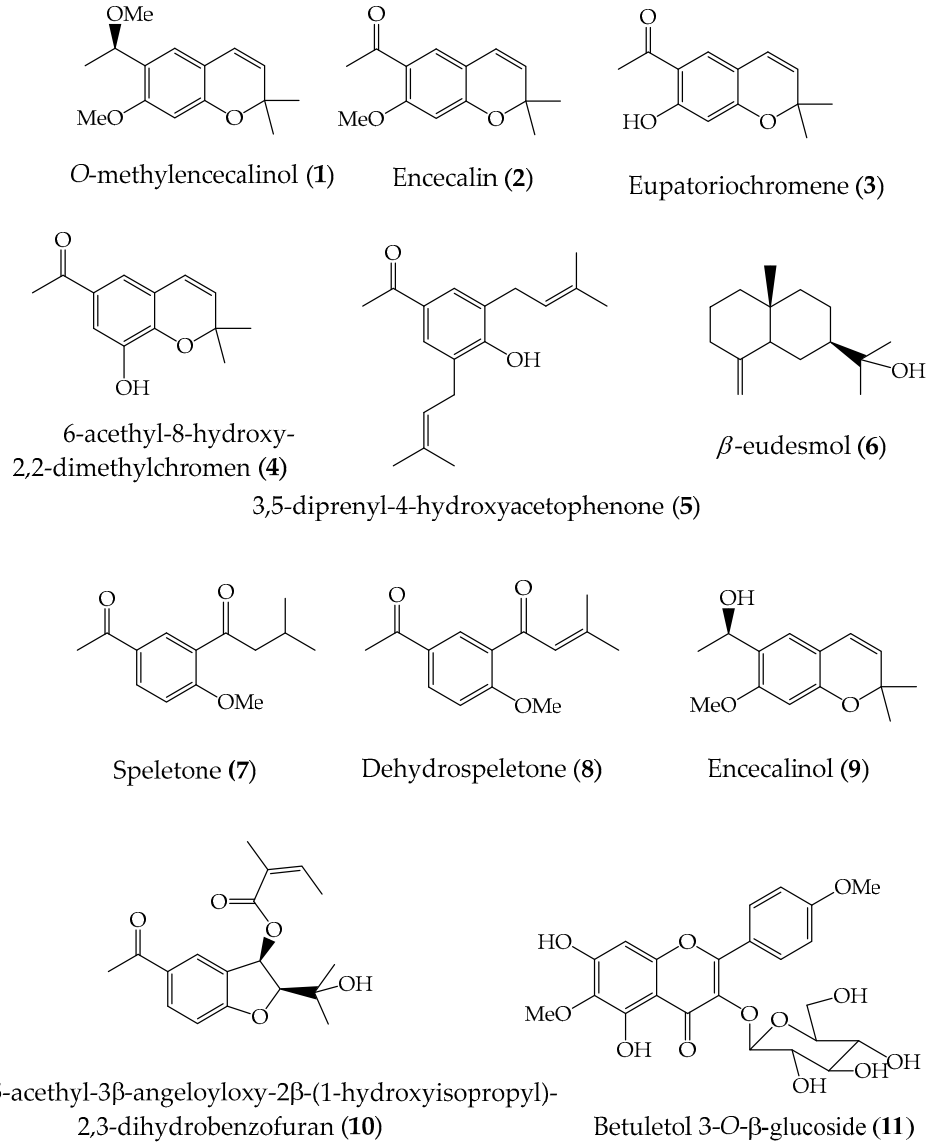

Figure 1. Compounds isolated from aerial parts of *A. pichinchensis*.

Since there is little evidence on the chemical characterization of compound **11**, we will provide more information on its structural elucidation in this study. Compound (**11**) was obtained as a yellow-colored amorphous solid; the analysis of the data obtained by ^{1}H, ^{13}C, and DEPT NMR (S1, S2, and S3), the two-dimensional heteronuclear HMBC experiment (S4) as well as in FAB+ mode mass spectrometry (spectrum 5) gave evidence on the chemical structure of the obtained solid. In the hydrogen NMR spectrum, two double signals are observed at (δ 6.88 and δ 8.12), which show ortho-type coupling constants (J = 8.7 Hz and 8.9 Hz) which are characteristic of the symmetric hydrogens that are in a 1,4. -disubstituted aromatic system, in addition to these signals, a single signal is observed at δ 6.9 and that is assigned to a pentasubstituted benzene. At δ 5.44 another double signal appears (J= 7.7 Hz), as well as several signals between δ 3.5 and δ 4.5 characteristic of a glucopyranose; These signals are evidence in the 13C NMR spectrum, indicating that the aglycone is glycosylated. Once these signals were assigned, it was established that it was a glycosylated flavonol and that, based on the HMBC experiment (S5), the sugar molecule is connected to the oxygen in the 3-position of the aglycone. Once the structural identity was established, the yellow solid was identified as betuletol 3-O-β-glucoside.

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3.2. Inhibition of LPS-induced NO production by compounds 1-11

The evaluation of cytotoxicity in RAW 264.7 cells by MTS indicated that up to 75 microg/mL for 48 h of incubation, compounds **1-5** and **7-11** did not affect cell viability. After treatment with LPS (1 μg/mL) for 24 h, the concentration of nitrite in the medium increased markedly. When RAW 264.7 cells were treated with different concentrations of compounds **1** to **5** and **7** to **11**, a significant inhibition by compound **11** on nitrite production was detected as a concentration-dependent response. The IC_{50} values of compounds **1-11** to inhibit LPS-induced NO production are presented in Table 1.

Table 1. Percentage of inhibition of NO production and cell viability in RAW 264.7 macrophages at a concentration of 75 μM of compounds **1-11.**

Compounds	Cell viability (%)[a]	NO Inhibition (%)	NO inhibition (IC_{50}, μM)
1	109.9 ± 7.16	0.95 ± 1.35	> 75
2	112.4 ± 24.08	16.75 ± 5.36	> 75
3	115.6 ± 1.58	11.98 ± 7.85	> 75
4	99.33 ± 12.39	22.63 ± 10.38	> 75
5	100.20 ± 2.95	29.77 ± 18.27	> 75
6	61.14 ± 6.31	-----	-----
7	104.7 ± 1.82	5.90 ± 8.35	> 75
8	103.9 ± 3.83	36.73 ± 16.93	> 75
9	110.9 ± 8.3	29.77 ± 9.37	> 75
10	121.2 ± 10.20	5.98 ± 5.22	> 75
11	101.3 ± 1.62	75.08 ± 3.07	20.55 ± 0.27
DMSO[b]	-----	-----	-----
Indomethacin[c] (84 μM)	-----	65.93 ± 6.03	54.69 ± 10.34
Etoposide[d] (68 μM)	42.02 ± 4.23	-----	-----

[a] Cell viability at 75 μM. [b] Blank control. [c] Positive control for NO production assay. [d] Positive control for cytotoxicity against RAW 264.7 cell.

The cell viability assay showed that, except for compound **6** (61.14%), all the compounds tested at concentrations of 9.4, 18.8, 37.5 and 75 μM did not affect the viability of macrophages and showed survival rates greater than 90%. Based on these results, except compound **6** (37.5 μM), the compounds were tested at 75 μM to evaluate their effect on nitric oxide production in RAW 264.7 cells stimulated with LPS. Results showed that only compound **11** inhibited the production of NO in a dose-dependent manner (Figure 2a), with an IC_{50} value of 20.55 ± 0.27 μM. This compound turned out to be more active than indomethacin, since the maximum evaluated concentration (75 μM) inhibited by 75.08 ± 3.07 % NO production, and indomethacin inhibited by 65.93 ± 6.03 at the concentration of 84 μMThis effect was not due to cytotoxicity, since it did not affect cell viability of the RAW 264.7 cells up to a concentration of 75 μM. Inflammation is a defense mechanism against external stimuli such as infections (bacteria) or internal stimuli inherent to living organisms (stress); however, excessive inflammation can cause chronic inflammatory problems. Some researchers indicate that resident macrophages are key in the Inflammation cascade, which is why the inhibition of inflammatory mediators or the NF-kB signaling pathway in macrophages can be useful in the development of therapies aimed at specific pro-inflammatory [61]. Also studied was the effect of compound **11** on the expression of cytokine IL6 y factor nuclear kappa B (KF-kB). Per the previous results, the effect of compound **11** on the secretion of the proinflammatory cytokines IL-6 and NF-kβ are shown in Figures

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2b and **2c**. As mentioned before, it is well known that in the inflammatory process, the anti- and pro-inflammatory cytokines play an important role in the activation, maintenance, and regulation of inflammation [40–43].

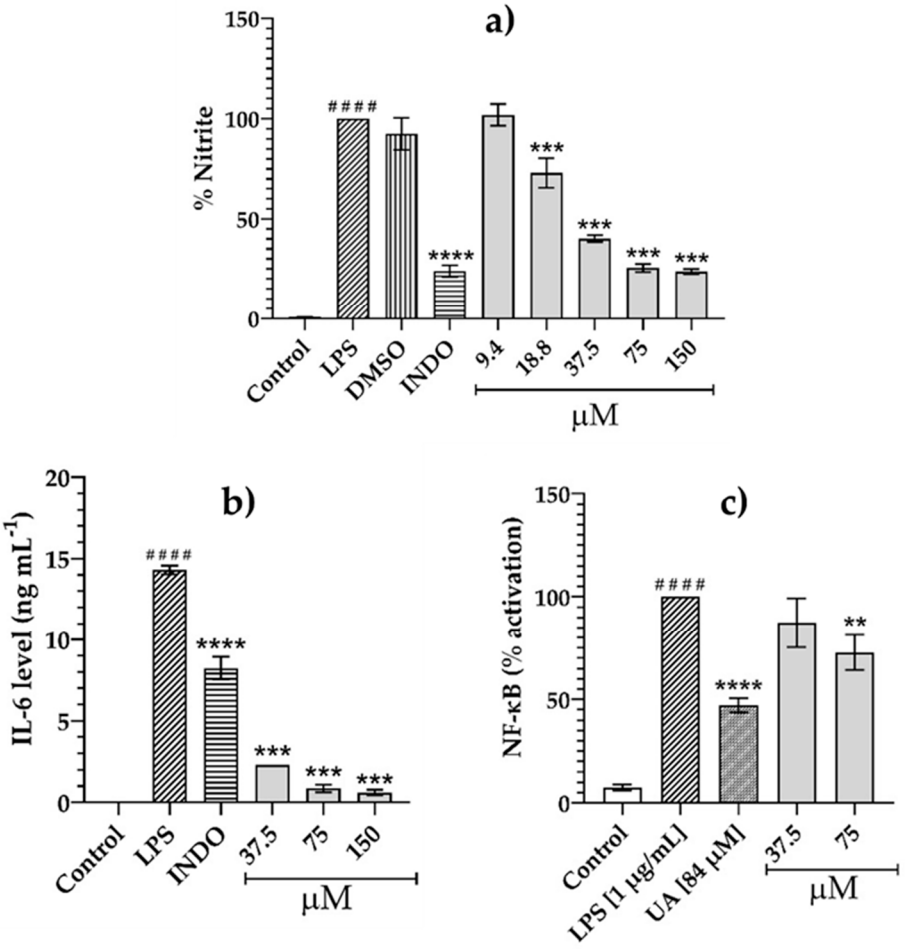

Figure 2. Effect of compound **11** on a) NO, b) IL-6 and c) NF-kβ of RAW 264.7 macrophages activated with LPS. Data are expressed as mean ± SD values of experiments in triplicate independent assays. ** $p < 0.01$, *** $p < 0.001$ vs LPS-treated cells; #### $p < 0.001$ vs vehicle control.

The compound **11**, like some essential oils, showed an anti-inflammatory effect demonstrated by the inhibition of nitric oxide associated with regulating the expression of pro-inflammatory cytokines [63–65]. Other compounds have shown similar effects to compound (**11**) in the same inflammation model, such as the case of the compound 4-methoxycinnamyl *p*-coumarate isolated from the species *Etlingera pavieana*, which revealed an IC_{50} of 15.0 ± 1.4 μM [66]. On the other hand, the triterpene methyl lucidenate L isolated from the species *Ganoderma lucidum* exhibited an IC_{50} of 36.8 ± 1.0 μM [67]. It should be noted that compound (**11**) has not been reported in Ageratina species, although it has been reported in species of the Asteraceae family, for example, in the species *Arnica montana* and *Arnica chamissonis*, both plants have been used in traditional medicine as a healing and anti-inflammatory agent at the extract level [55,69]. These species are characterized by containing sesquiterpene lactones such as helenalin, 11α,13-dihydrohelenalin, and chamissonolide, which inhibit activation of transcription factor NF-κβ [70].

Regarding compound (**6**), it was not evaluated to determine its effect on nitric oxide secretion, this due to the cell viability assay having a survival rate of RAW 264.7 macrophages of less than 90% (61.14 ± 6.31%) at the highest concentration evaluated, however, at a concentration of 37 mM it does not inhibit cell viability (104.00 ± 3.64 μM). β-Eudesmol is a sesquiterpene that has already been isolated from several species, to mention a few, there are found *Atracrylodes lancea* [57], *Zingiber*

Zerumbet [71], *Guttería friesiana* [72] and *Murraya tetramera* [73]. Likewise, several biological effects have been demonstrated for this secondary metabolite, of which we can highlight its anti-inflammatory effect, as a tumor suppressor, and anticancer [71–73]. The above agrees with the inhibitory effect that compound (**6**) showed on cell viability.

4. Conclusions

The inhibitory effect on NO production in an in vitro inflammatory model was evaluated of compounds (**1-5**, **7-11**) isolated from leaves and flowers of *A. pichinchensis*. Among these, the betuletol 3-*O*-β-glucoside (**11**) inhibited the production of NO in a dose-dependent manner, in particular, treatment with 75 μM significantly decreased NO production by 75.08 ± 3.07 % (IC_{50} = 20.55 ± 0.27). The effect demonstrated by the inhibition of nitric oxide associated with the regulation of the expression of proinflammatory cytokines (IL-6) and inhibition of NF-kβ activation indicates that compound **11** may be useful as a therapeutic agent in the treatment of inflammation-related diseases caused by the overactivation of macrophages.

Supplementary Materials: Effect of compounds (**1-11**) on cell viability and NO production in RAW 264.7 cells, at different concentrations, tables 1 and 2. ^{1}H and ^{13}C NMR spectra of compounds (**1-6** and **9-10**) Figures S1 -S12 and S15-S18, ^{1}H NMR spectra of compounds (**7-8**) Figure S13-S14, 1D and 2D NMR spectra of compound (**11**), Figures S19-S22 and Mass-Mass Spectrum (FAB+), Figure S23.

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